Journal of Neuroscience Research

Journal of Neuroscience Research 88:2618–2629 (2010)

Potential In Vivo Amelioration by N-Acetyl-L-Cysteine of Oxidative Stress in Brain in Human Double Mutant APP/PS-1 Knock-In Mice: Toward Therapeutic Modulation of Mild Cognitive Impairment

Quanzhen Huang,[1,2,3] Christopher D. Aluise,[1,2,3] Gururaj Joshi,[1,2,3] Rukhsana Sultana,[1,2] Daret K. St. Clair,[4] William R. Markesbery,[3] and D. Allan Butterfield[1,2,3]★

[1]Department of Chemistry, University of Kentucky, Lexington, Kentucky
[2]Center of Membrane Sciences, University of Kentucky, Lexington, Kentucky
[3]Sanders-Brown Center on Aging, University of Kentucky, Lexington, Kentucky
[4]Department of Toxicology, University of Kentucky, Lexington, Kentucky

Alzheimer's disease (AD) is the most prevalent form of dementia among the elderly. Although the underlying cause has yet to be established, numerous data have shown that oxidative stress is implicated in AD as well as in preclinical stages of AD, such as mild cognitive impairment (MCI). The oxidative stress observed in brains of subjects with AD and MCI may be due, either fully or in part, to increased free radicals mediated by amyloid-β peptide (Aβ). By using double human mutant APP/PS-1 knock-in mice as the AD model, the present work demonstrates that the APP/PS-1 double mutation results in elevated protein oxidation (as indexed by protein carbonyls), protein nitration (as indexed by 3-nitrotyrosine), as well as lipid peroxidation (as indexed by protein-bound 4-hydroxy-2-nonenal) in brains of mice aged 9 months and 12 months. APP/PS-1 mice also exhibited lower levels of brain glutathione peroxidase (GPx) in both age groups studied, whereas glutathione reductase (GR) levels in brain were unaffected by the mutation. The activities of both of these antioxidant enzymes were significantly decreased in APP/PS-1 mouse brains, whereas the activity of glucose-6-phosphate dehydrogenase (G6PDH) was increased relative to controls in both age groups. Levels of peptidyl prolyl isomerase 1 (Pin1) were significantly decreased in APP/PS-1 mouse brain aged 9 and 12 months. Administration of N-acetyl-L-cysteine (NAC), a glutathione precursor, to APP/PS-1 mice via drinking water suppressed increased protein oxidation and nitration and also significantly augmented levels and activity of GPx in brain from both age groups. Oral administration of NAC also increased the diminished activity of GR and protected against lipid peroxidation in brains of 9-month-old APP/PS-1 mice only. Pin1 levels, GR levels, and G6PDH activity in brain were unaffected by oral administration of NAC in both age groups. These results are discussed with reference to the therapeutic potential of this brain-accessible glutathione precursor in the treatment of MCI and AD.

Key words: APP/PS-1; glutathione; N-acetyl cysteine; protein oxidation; lipid peroxidation; oxidative stress; Alzheimer's disease

Alzheimer's disease (AD) affects more than 5 million Americans, including 50% of people aged 85 years or older. There is no effective therapy for the treatment of this dementing disorder. Pathological characteristics of AD include the presence of senile plaques composed of a core of aggregated amyloid-β peptide (Aβ) surrounded by dystrophic neurites, neurofibrillary tangles composed of hyperphosphorylated tau protein, and synapse loss (Katzman and Saitoh, 1991; Bosetti et al., 2002). Pathophysiologically, a free radical-mediated oxidative stress

The first three authors contributed equally to this work.

Contract grant sponsor: NIH; Contract grant number: AG-10836; Contract grant number: AG-05119.

★Correspondence to: Prof. D. Allan Butterfield, Department of Chemistry, Center of Membrane Sciences, and Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40506. E-mail: dabcns@uky.edu

Received 24 November 2009; Revised 5 March 2010; Accepted 16 March 2010

Published online 1 June 2010 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.22422

hypothesis for AD has gained considerable attention (Hensley et al., 1995; Markesbery and Lovell, 1998; Butterfield et al., 2001; Butterfield and Lauderback, 2002). In AD brain, oxidative stress is indexed by significant protein oxidation (Butterfield and Lauderback, 2002), DNA and RNA oxidation (Mecocci et al., 1993; Lovell et al., 1999; Nunomura et al., 1999; Wang et al., 2005), lipid peroxidation (Markesbery and Lovell, 1998; Lauderback et al., 2001), and neuronal dysfunction or death. Increased production of reactive oxygen species (ROS) along with depletion of endogenous antioxidants is observed in AD (Beckman and Ames, 1998). Consistently with this observation, α-tocopherol (vitamin E) administration to AD patients delayed disease progression compared with placebo-treated subjects (Sano et al., 1997), suggesting that oxidative stress-mediated AD pathogenesis conceivably can be altered by antioxidant therapy.

It has been well documented that Aβ(1–42) is involved in AD pathogenesis. Our laboratory was instrumental in demonstrating that Aβ(1–42) mediated oxidative stress in brain in vivo (Butterfield et al., 2001). Based on these studies, a mouse model showing accelerated human Aβ(1–42) deposition in brain has been used to study the oxidative stress parameters and potentials of various antioxidants as therapeutics for mild cognitive impairment (MCI), arguably the earliest form of AD itself. This mouse model, commonly referred to as the *APP/PS-1 model*, uses knock-in technology to humanize the mouse Aβ sequence (NLh) and create a P to L mutation of codon 264 of presenilin-1 (PS1), a mutation identified in familial AD (FAD; Reaume et al., 1996; Siman et al., 2000). APP/PS-1 mice showed increased Aβ production and accelerated amyloid deposition in the brain (Borchelt et al., 1997; Anantharaman et al., 2006). These mice demonstrate Aβ deposits at 9 months of age and frank plaques at 12 months of age (Anantharaman et al., 2006). Previous studies from our laboratory have shown that brains from APP/PS-1 mice have increasingly elevated oxidative stress over ages of 1–15 months (Abdul et al., 2008). In addition, neurons from APP/PS-1 mice have increased basal protein oxidation and lipid peroxidation and are more vulnerable to oxidation by exogenous oxidants compared with neurons from wild-type mice (Mohmmad Abdul et al., 2006). These studies suggest that APP/PS-1 mice can be used as a model of FAD, which is characterized by Aβ(1–42)-mediated oxidative stress (Butterfield et al., 2006a).

Consistent with oxidative stress, depletion of glutathione (GSH), an intracellular antioxidant, is known to be involved in several neurodegenerative disorders, including AD (Benzi and Moretti, 1995; Markesbery, 1997; Butterfield et al., 2002). Decreased intracellular GSH levels or decreased GSH accompanied by a concomitant increase in the oxidized form of GSH, glutathione disulfide (GSSG), may index and/or cause oxidative stress. Increased levels of endogenous GSH by dietary or pharmacological intake of GSH precursors or mimetics protect the brain against oxidative stress (Anderson and Luo, 1998; Pocernich et al., 2000, 2001; Halliwell, 2001; Butterfield et al., 2001; Butterfield, 2002; Ansari et al., 2006). Considering the importance of developing new antioxidant compounds and the relevance of their application in the treatment of neurodegenerative disease, we focused our attention on the glutathione precursor N-acetyl cysteine (NAC).

NAC is an FDA-approved drug typically used in the treatment of acetaminophen-mediated liver toxicity (Prescott et al., 1977). NAC provides cysteine, the rate-limiting substrate in GSH synthesis (Dringen and Hamprecht, 1999). NAC can act as an antioxidant by raising intracellular levels of cysteine or by direct scavenging of ROS. NAC significantly increased endogenous GSH levels in vivo in cortical synaptosome cytosol (Pocernich et al., 2000, 2001). Previous studies from our laboratory have shown that NAC can partially protect brain-derived membrane proteins against peroxynitrite-induced damage (Koppal et al., 1999). In another study, NAC protected the brain against oxidative damage induced by 3-nitropropionic acid (Fontaine et al., 2000). In age-accelerated SAMP8 mice, NAC reverses memory impairment and diminishes brain oxidative stress (Farr et al., 2003). Apart from its antioxidant properties, NAC has been shown to improve neuron survival in the CA1 region of the hippocampus following ischemic-reperfusion injury (Zhang et al., 2003). NAC can also function as a transcription factor and can rescue neurons from apoptotic death by activation of the Ras-ERK pathway (Yan and Greene, 1998). NAC reduces inflammatory symptoms in brain by direct inhibition of nuclear factor-κB and blocking production of nitric oxide (NO) from both inducible nitric oxidase synthase (iNOS) and inflammatory cytokines (Pahan et al., 1998).

The current study was designed to determine whether in vivo NAC would protect the brain from APP/PS-1 knock-in mice against human Aβ-mediated oxidative stress. NAC was orally administered to 4- and 7-month-old mice for 5 months each to determine whether earlier antioxidant intervention would be advantageous compared with later administration. The time points were selected to test the hypothesis that NAC would be more effective against oxidative stress if administered prior to Aβ deposition compared with prior to formation of amyloid plaques.

MATERIALS AND METHODS

Chemicals

All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless stated otherwise. The OxyBlot kit used for protein carbonyl determination was purchased from Intergen (Purchase, NY). The primary antibody for the lipid peroxidation product, 4-hydroxy-2-nonenal (HNE), bound to proteins was purchased from Alpha Diagnostics (San Antonio, TX). The primary antibody for GPx was purchased from Chemicon (Temecula, CA). Transfer membranes for Western

blot and slot blot were purchased from Bio-Rad (Hercules, CA).

Animals

For this study, male wild-type (WT) and APP/PS-1 mice, approximately 30 g in size, housed in the University of Kentucky Central Animal Facility under 12-hr light/dark conditions and fed standard Purina rodent laboratory chow ad libitum were used. The animal protocols were approved by the University of Kentucky Animal Care and Use Committee. The APP/PS-1 mice used were the $APP^{NLh}/APP^{NLh} \times PS\text{-}1^{P264L.}/PS\text{-}1^{P264L.}$ double mutant generated by using the Cre-loc knock-in technology (Cephalon, Westchester, PA) to humanize the mouse Aβ sequence and to create a PS-1 mutation identified in human FAD (Reaume et al., 1996; Siman et al., 2000). These mice yield proper cleavage of APP to produce human Aβ. Gene expression as a result of crossing APP (NLh) and PS-1 (P264L) knock-in mice is driven by endogenous promoters of the APP and PS-1 genes; therefore, expression is limited by replacement of these genes and not by the expression of multiple transgenes (Anantharaman et al., 2006).

Treatments

Mice were divided into four groups. A group of WT and APP/PS-1 mice received drinking water, and another group of WT and APP/PS-1 mice received drinking water containing NAC (2 mg/kg/day) for a period of 5 months. A 0.001% solution of NAC in water (pH adjusted to 7.2 by NaOH) was prepared and given to mice. Water was changed every other day for 5 months. Approximately 4–5 ml of water was consumed per mouse per day, which gives a cumulative dose of 2 mg/kg per mouse per day, not accounting for spills. The NAC treatment was started at 4 months of age or at 7 months of age. The dose of NAC was chosen based on prior studies (Andreassen et al., 2000). After 5 months of NAC treatment, mice were euthanized, and brains were isolated and flash frozen in liquid nitrogen.

Preparation of Brain Homogenate

Brains were thawed and placed in ice-cold lysing buffer containing 4 μg/ml leupeptin, 4 μg/ml pepstatin, 5 μg/ml aprotinin, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ethylene glycol-bistetraacetic acid (EGTA), and 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.4. The brains were homogenized by 20 passes of a Wheaton tissue homogenizer, and the resulting homogenate was centrifuged at 2,000*g* for 10 min to remove debris. An aliquot of whole-brain homogenate was taken for slot blot analysis, whereas the supernatant was used for enzyme activity assays and protein level determination. Homogenized samples were assayed for protein concentration by the Pierce BCA method.

Protein Carbonyls

Samples (5 μl) of brain homogenate, 12% sodium dodecyl sulfate (SDS; 5 μl), and 10 μl of 10 times diluted 2,4-dinitrophenylhydrazine (DNPH) from 200 mM stock were incubated at room temperature for 20 min, followed by neutralization with 7.5 μl neutralization solution (2 M Tris in 30% glycerol). Protein (250 ng) was loaded in each well on a nitrocellulose membrane under vacuum using a slot blot apparatus. The membrane was blocked in blocking buffer (3% bovine serum albumin) in PBS 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 for 1 hr and incubated with a 1:100 dilution of anti-DNP polyclonal antibody in PBS containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 for 1 hr. The membrane was washed in PBS following primary antibody incubation three times at intervals of 5 min each. The membrane was incubated after washing with an anti-rabbit IgG alkaline phosphatase secondary antibody diluted in PBS in a 1:8,000 ratio for 1 hr. The membrane was washed three times in PBS for 5 min each and developed with Sigma fast tablets (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate [BCIP/NBT substrate]). Blots were dried, scanned in Adobe Photoshop, and quantified in Scion Image (PC version of Macintosh-compatible NIH image). No nonspecific binding of antibody to the membrane was observed.

Protein-Bound HNE and 3NT

Samples (5 μl) of brain homogenate, 12% SDS (5 μl), and 5 μl modified Laemmli buffer containing 0.125 M Tris base, pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol were incubated for 20 min at room temperature and were loaded (250 ng) in each well on a nitrocellulose membrane in a slot blot apparatus under vacuum. The membrane was treated as described above and incubated with a 1:5,000 dilution of anti-protein-bound HNE polyclonal antibody or 1:2,000 3NT antibody in PBS for 1 hr 30 min. The membranes were further developed and quantified as described above. A faint background staining resulting from the antibody alone was observed, but, because each sample had a control, this minor effect was controlled.

Western Blots

Samples (100 μg from cytosolic fraction) were incubated with sample loading buffer, and protein samples were denatured and electrophoresed on a 12.5% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane at 90 mA/gel for 2 hr. The blots were blocked for 1 hr in fresh wash buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, pH 7.4, containing 3% bovine serum albumin) and incubated with a 1:1,000 dilution of GPx, GR, or Pin1 monoclonal antibody in PBS for 1 hr. The membrane was washed three times in PBS and was incubated for 1 hr with an anti-rabbit IgG alkaline phosphatase secondary antibody diluted in PBS in 1:8,000 ratio. The membrane was washed for three times in PBS for 5 min and developed with Sigma fast tablets (BCIP/NBT substrate). In all cases, nonspecific background labeling by secondary antibody was negligible. Blots were dried, scanned, and quantified in Scion Image.

Enzyme Activity Assay

GPx activity. GPx activity was measured by using a reaction mixture consisting of 0.2 mM H_2O_2, 1.0 mM GSH, 0.14 U of glutathione reductase (GR), 1.5 mM NADPH,

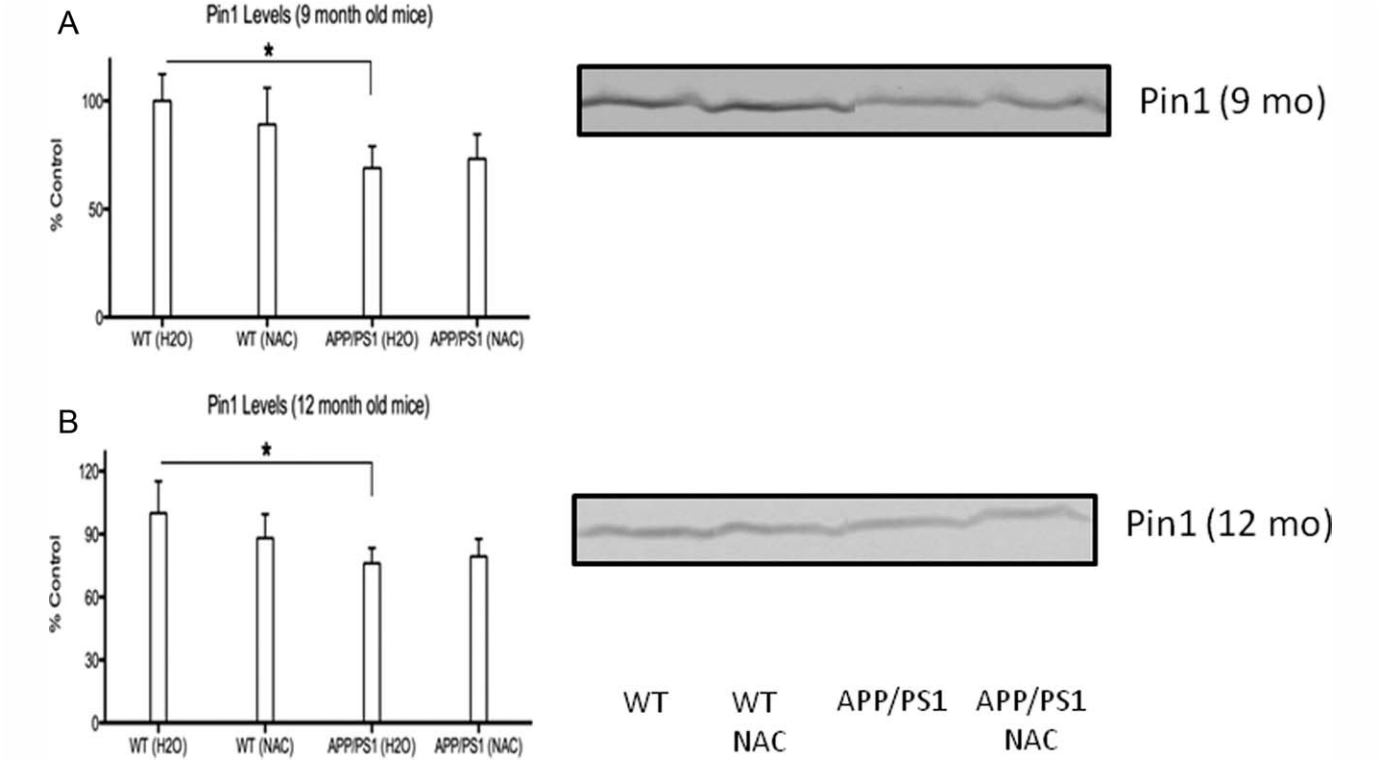

Fig. 1. Pin1 protein levels in WT and APP/PS1 mice aged 9 months (**A**) and 12 months (**B**) orally given NAC and normal drinking water. Brain isolated from APP/PS1 mice given normal drinking water had significantly lower levels of Pin1 compared with WT mice given normal drinking water in both age groups. Administration of NAC to mice in both age groups had no effect on levels of this protein in brain. Mean ± SEM, $\star P < 0.05$, n = 9 animals per group.

1.0 mM sodium azide, and 0.1 M phosphate buffer (pH 7.4) and 1 mg/ml of supernatant protein (Wheeler et al., 1990). The changes in absorbance were recorded at 340 nm in a 96-well microtiter plate, and enzyme activity was calculated as nmol NADPH oxidized min^{-1} mg^{-1} protein.

GR activity. GR activity was measured by using a reaction mixture consisting of 0.1 M phosphate buffer (pH 7.6), 0.5 mM EDTA, 1.0 mM oxidized glutathione, 0.1 mM NADPH, and 10 μl PMS in a total volume of 200 μl (Carlberg and Mannervik, 1985). The enzyme activity was assayed in a 96-well plate reader by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized min^{-1} mg^{-1} protein.

Glucose-6-phosphate dehydrogenase activity. Glucose-6-phosphate (G6PDH) was measured by using a standard assay mixture [Tris-HCl buffer (0.05 M) pH 7.6, $NADP^+$ (0.1 mM), $MgCl_2$ (8 mM), and glucose-6-phosphate (0.8 mM)]. The reaction was monitored in a 96-well plate reader by measuring the increase of absorbance at 340 nm.

Statistical Analysis

Two-way ANOVA followed by a multiple-comparisons test (Tukey HSD) was used to assess statistical significance. $P < 0.05$ was considered significant.

RESULTS

NAC Effects on Levels of Pin1 in 9- and 12-Month-Old WT and APP/PS-1 Mice

Expression levels of Pin1 in 9- and 12-month-old WT and APP/PS-1 mice are shown in Figure 1. APP/PS-1 mice given normal drinking water had significantly decreased levels of Pin1 in brain compared with WT mice in both age groups. Administration of NAC in drinking water of APP/PS-1 mice slightly enhanced the levels of Pin1 in these mouse brains relative to brains from APP/PS-1 mice given normal water; however, this result was not significant in either age group. NAC had no significant effect on Pin1 levels in brain from WT mice.

Effect of NAC on Protein Oxidation and Lipid Peroxidation in Brains Isolated From APP/PS-1 Mice

Protein carbonyls and protein-bound HNE are indices of oxidative damage to proteins and lipids, respectively (Butterfield and Stadtman, 1997). Figures 2 and 3 show the levels of protein carbonyls and protein-bound HNE in brains from both 9- and 12-month-old WT and APP/PS-1 mice of all treatment groups. Confirming our previous studies (Abdul et al., 2008), there

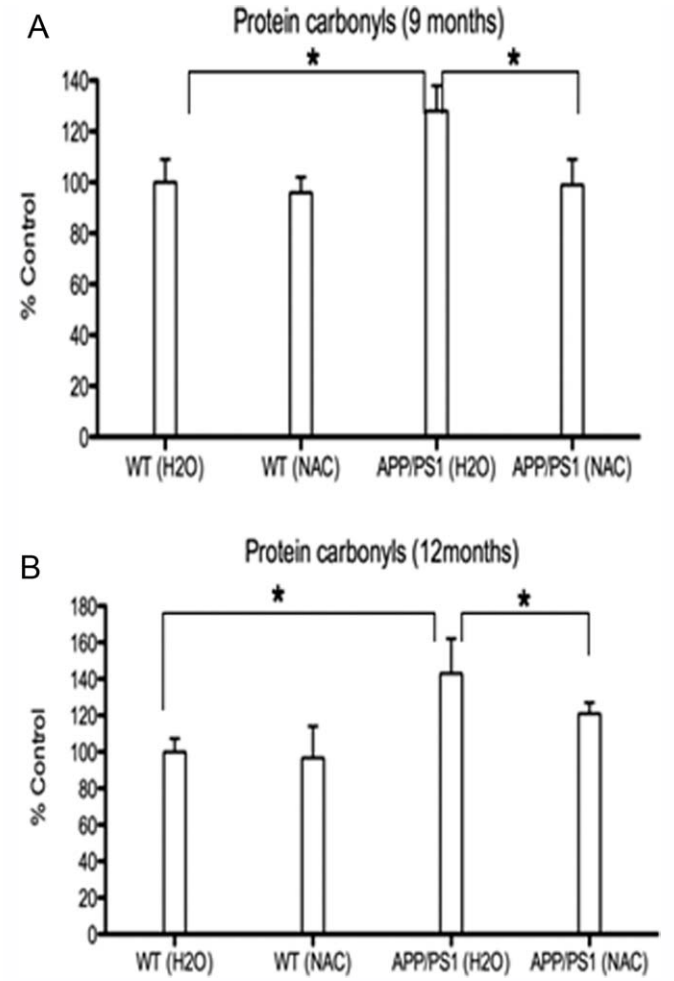

Fig. 2. Levels of protein carbonyls in brain isolated from WT and APP/PS1 mice aged 9 months (**A**) and 12 months (**B**) given normal drinking water and water containing NAC. In both age groups studied, APP/PS1 mice given normal drinking water had significantly higher levels of protein carbonyls than WT mice given normal water. Addition of NAC to drinking water of APP/PS1 mice significantly suppressed levels of protein carbonyls in brain of APP/PS1 mice in both age groups. Mean ± SEM, $\star P < 0.05$ n = 9 animals per group.

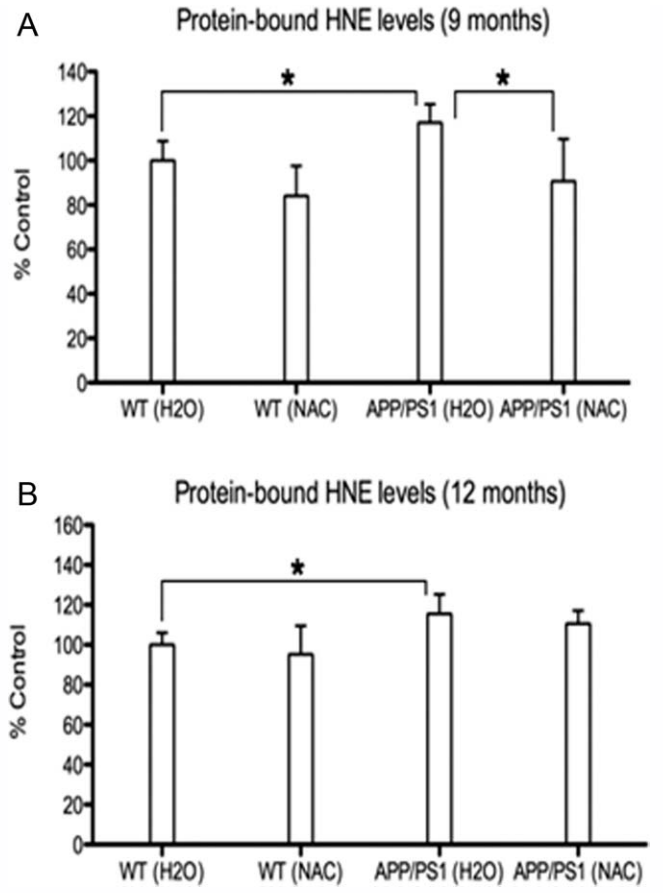

Fig. 3. Levels of protein-bound HNE in brain isolated from WT and APP/PS1 mice aged 9 months (**A**) and 12 months (**B**) given normal drinking water and water containing NAC. In both age groups studied, APP/PS1 mice given normal drinking water had significantly higher levels of protein-bound HNE than WT mice given normal water. Addition of NAC to drinking water of APP/PS1 mice significantly suppressed levels of protein-bound HNE in brain of APP/PS1 mice in the 9-month-old group only. Mean ± SEM, $\star P < 0.05$ n = 9 animals per group.

was a significant elevation in protein carbonyls and HNE-bound proteins in brains isolated from APP/PS-1 mice that were given normal water compared with the levels in brain isolated from WT mice given normal water ($P < 0.05$) in both age groups. Administration of water containing NAC to APP/PS-1 mice significantly reduced the levels of brain protein carbonyls compared with brain isolated from APP/PS-1 mice given normal drinking water in both the 9- and the 12-month-old mice ($P < 0.05$). Nine-month-old APP/PS-1 mice given NAC also had significantly lower HNE bound proteins in brain compared with APP/PS-1 mice given normal drinking water; however, this effect was not observed in 12-month-old mice. NAC had no significant effect on these parameters in brains from WT mice.

Effect of NAC on Protein Nitration in Brains Isolated From APP/PS-1 Mice

3NT is an index of nitrosative stress. Levels of this protein modification are increased in brains from MCI and AD patients compared with controls (Smith et al., 1997; Castegna et al., 2003; Sultana et al., 2006a, 2007a,b; Butterfield et al., 2007b). Figure 4 shows the levels of 3NT in brains from both 9- and 12-month-old APP/PS-1 and WT mice. There was significant elevation of 3NT in brains isolated from APP/PS-1 mice that were given normal water compared with the levels in brain isolated from WT mice given normal water ($P < 0.05$) in both age groups. Administration of water containing NAC to APP/PS-1 mice significantly reduced 3NT levels in brain compared with APP/PS-1 mice given normal drinking water in both the 9- and the 12-

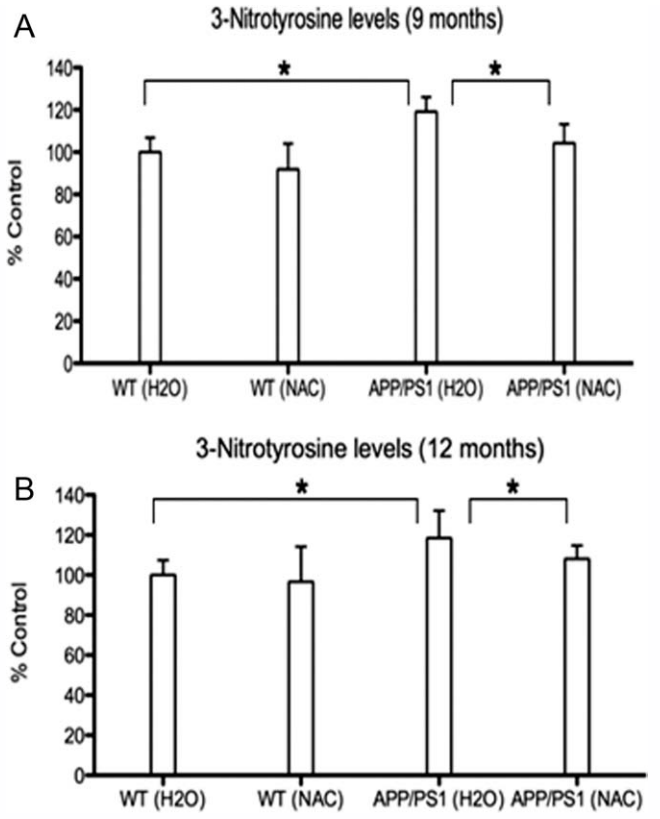

Fig. 4. Levels of 3NT in brain isolated from WT and APP/PS1 mice aged 9 months (**A**) and 12 months (**B**) given normal drinking water and water containing NAC. In both age groups studied, APP/PS1 mice given normal drinking water had significantly higher levels of 3NT than WT mice given normal water. Addition of NAC to drinking water of APP/PS1 mice significantly suppressed levels of 3NT in brain of APP/PS1 mice in both age groups studied. Mean ± SEM, $\star P < 0.05$ n = 9 animals per group.

month-old age groups ($P < 0.05$). NAC had no significant effect on 3NT levels in brains from WT mice.

Effects of NAC Treatment on Antioxidant Enzyme Levels and Activities in Brains Isolated From APP/PS-1 Mice

Oxidative stress inherently results in, or is predicated by, an alteration in antioxidants and antioxidant enzymes (Butterfield and Stadtman, 1997). Studies have shown alterations in some antioxidant enzymes in the brain from subjects with AD (Lovell et al., 1995; Marcus et al., 1998) and MCI (Sultana et al., 2008). Figure 5 shows the activity and levels of brain GPx in the 9- and 12-month-old treatment groups. There was a significant reduction in both GPx levels and activity in brains isolated from APP/PS-1 mice that were given normal water compared with brains isolated from WT mice given normal water at each age examined ($P < 0.05$). Administration of water containing NAC to APP/PS-1 mice significantly augmented the protein levels and activity of GPx in brain relative to the levels in brain isolated from APP/PS-1 mice given drinking water devoid of NAC in both age groups ($P < 0.05$).

Brain GR activity for 9- and 12-month-old mice from all treatment groups is shown in Figure 6. The APP/PS-1 double mutation results in significantly decreased activity of GR in brain in both age groups; however, the protein level of GR was unaffected (data not shown). Oral administration of NAC significantly rescued GR activity in the 9-month-old APP/PS-1 mice compared with APP/PS-1 mice given normal water only. However, NAC treatment of APP/PS-1 mice initiated at 7 months did not alter the antioxidant activity of brain GR at 12 months compared with APP/PS-1 mice given drinking water without NAC (Fig. 6B). It was interesting to observe that NAC treatment also reduced GR activity in WT mice brain from 9- and 12-month-old animals (Fig. 6B).

G6PDH activity of both age groups is shown in Figure 7. G6PDH activity was significantly increased in APP/PS-1 mice brains compared with WT mice brains in both age groups (Fig. 7). Administration of NAC had no apparent effect on the activity of this enzyme in either 9- or 12-month-old APP/PS-1 and WT mice.

DISCUSSION

Impairment of the central nervous system (CNS) can often be caused by oxidative stress mechanisms, leading to the development of some neurodegenerative diseases. Oxidative stress is implicated in brains of subjects with MCI and AD (Smith et al., 1997; Markesbery, 1997; Butterfield et al., 2001, 2006a,c, 2007a,b; Lauderback et al., 2001; Keller et al., 2005). Aβ(1–42) is associated with increased free radical generation, leading to protein and lipid oxidation (Varadarajan et al., 2000; Butterfield et al., 2001; Lauderback et al., 2001; Butterfield and Lauderback, 2002). A comprehensive model for neurodegeneration in AD combining two established notions, viz. elevated oxidative stress in AD brain and the centrality of Aβ in the cause and consequences of this disorder, was developed in our laboratory (Varadarajan et al., 2000; Butterfield et al., 2001; Butterfield and Lauderback, 2002). Based on the substantial amount of data supporting the concept that oxidative stress is implicated in AD, treatment with brain-accessible antioxidants could be a promising approach to slow disease progression if given early enough in the disease.

Starting NAC treatment at 4 months and 7 months groups was chosen based on previous pathological studies. APP/PS-1 mice develop elevated levels of Aβ(1–42) at about 6 months of age (Borchelt et al., 1996; van Groen et al., 2006). Aβ(1–42) deposition is observed at 9 months (Anantharaman et al., 2006), but frank plaques are observed at 12 months of age (Anantharaman et al., 2006). We hypothesized, based on this observation, that administration of NAC at an early age (4 months old) for a period of 5 months would reduce Aβ(1–42)-mediated oxidative stress in APP/PS-1 mice at a later age (9 months old), when Aβ(1–42) begins to be deposited.

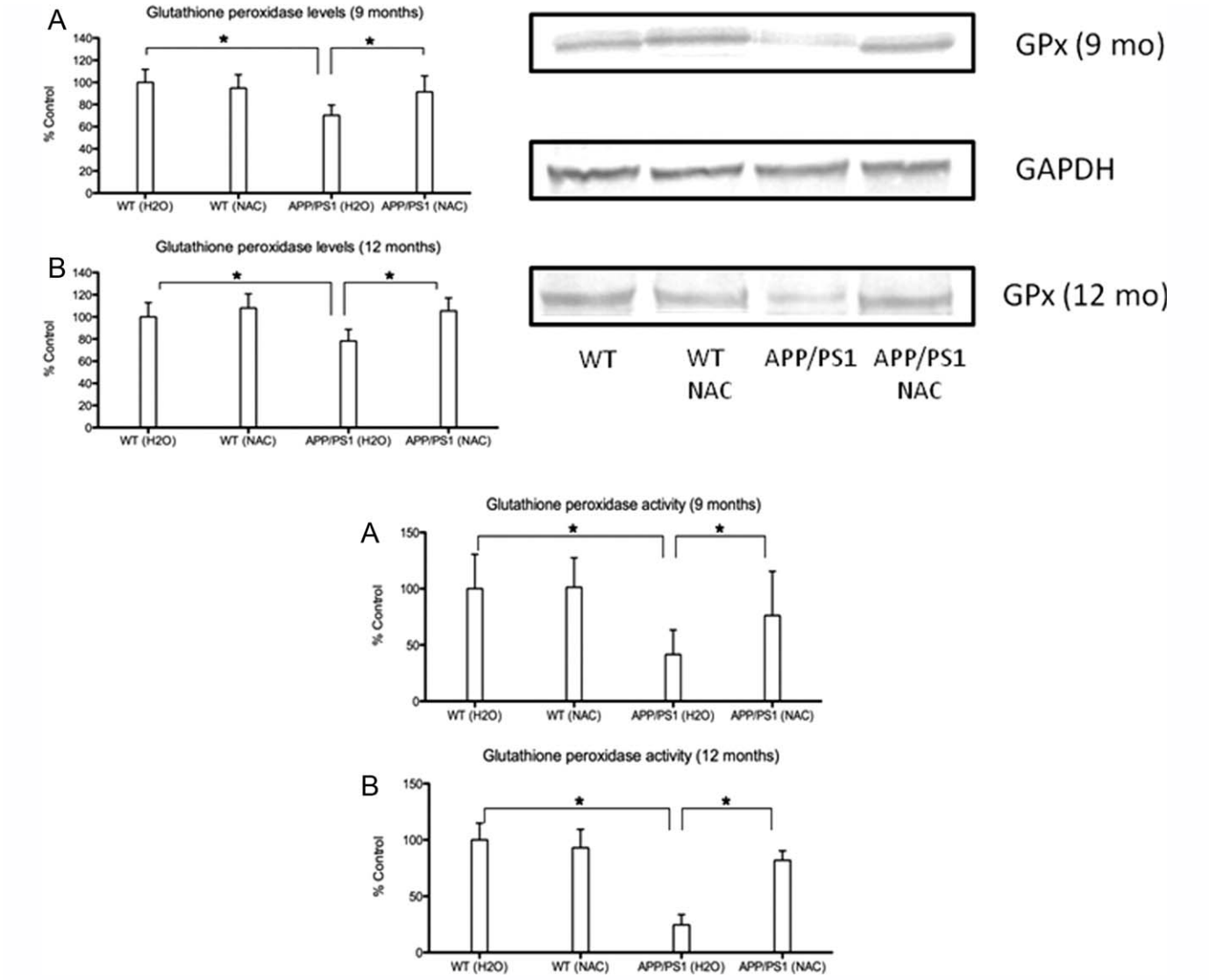

Fig. 5. **Top:** Levels of GPx in brain isolated from WT and APP/PS1 mice aged 9 months (**A**) and 12 months (**B**) given normal drinking water and water containing NAC. Equal amount of protein (100 μg) as assessed by the BCA assay was loaded per lane; GAPDH blot corresponding to 9 month group is shown as a representative loading control. In both age groups, APP/PS1 mice given normal water had significantly lower GPx activity than WT mice given normal water. A significant increase was observed in both age groups in GPx activity in brain isolated from APP/PS1 mice given NAC compared with APP/PS1 mice given normal water. Mean ± SEM, $\star P < 0.05$. **Bottom:** Also shown is activity of GPx in brain isolated from WT and APP/PS1 mice aged 9 months (**A**) and 12 months (**B**) given normal drinking water and water containing NAC. In both age groups, APP/PS1 mice given normal water had significantly lower GPx activity than WT mice given normal water. A significant increase was observed in both age groups in GPx activity in brain isolated from APP/PS1 mice given NAC compared with APP/PS1 mice given normal water. Mean ± SEM, $\star P < 0.05$, n = 9 animals per group.

We also posited that early administration of NAC (4 months old) would protect APP/PS-1 mice brains to a superior degree compared with initiation of NAC administration at a later age (7 months old), ending after the onset of significantly more Aβ deposition, i.e., plaque formation. Because clinical signs of memory impairment in AD patients are thought to be preceeded by 20 years of accumulated neuropathological changes, this study has implications for possible prevention of oxidative stress as a result of Aβ.

Aβ is produced by sequential cleavages of the amyloid precursor protein (APP) by β- and γ-secretase. Although the biochemical significance has yet to be established, processing of APP to Aβ occurs via the non-amyloidogenic and amylogenic pathways. One hallmark of AD pathology is significant amounts of senile plaques composed of Aβ(1–40) and Aβ(1–42), suggesting that the amyloidogenic pathway is up-regulated in AD. It has been suggested that Pin1 inhibition causes APP processing to follow the amylogenic pathway to produce more

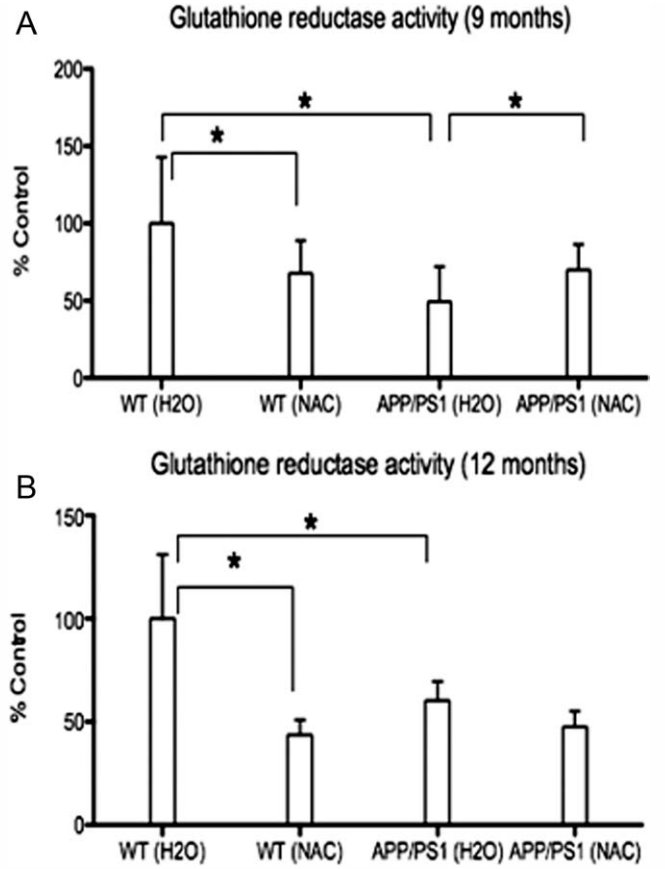

Fig. 6. Activity of GR in brain isolated from WT and APP/PS1 mice aged 9 months (**A**) and 12 months (**B**) given normal drinking water and water containing NAC. In both age groups, APP/PS1 mice given normal water had significantly lower GR activity than WT mice given normal water. A significant increase was observed in GR activity in brain isolated from APP/PS1 mice given NAC compared with APP/PS1 mice given normal water in the 9 month age group only. Mean ± SEM, $^{\star}P < 0.05$, n = 9.

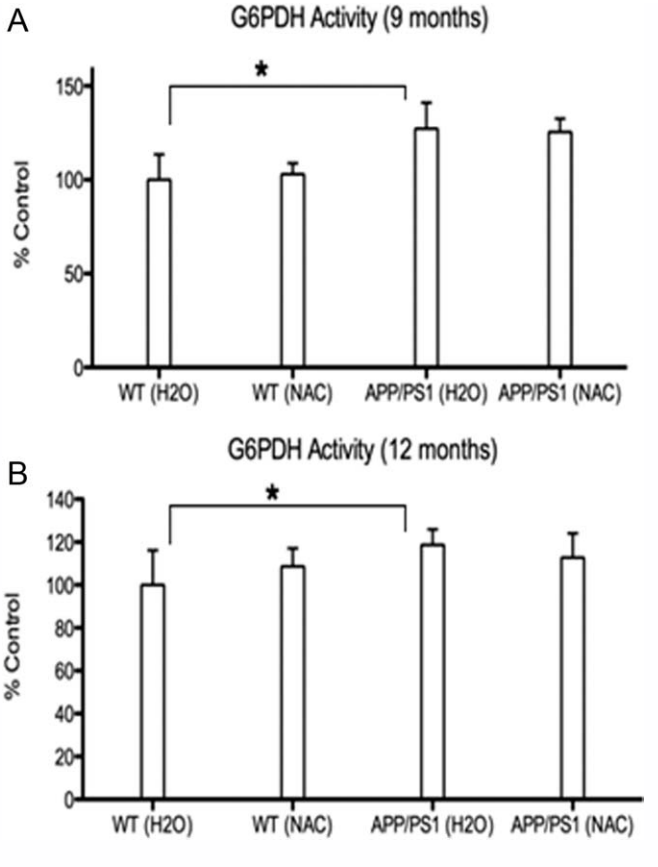

Fig. 7. Activity of G6PDH in brain isolated from WT and APP/PS1 mice aged 9 months (**A**) and 12 months (**B**) given normal drinking water and water containing NAC. In both age groups, a significant increase in G6PDH activity was observed in brain isolated from APP/PS1 mice given normal water compared with brain isolated from WT mice given normal water. Addition of NAC to drinking water of APP/PS1 mice had no apparent effect on the activity of this enzyme. Mean ± SEM, $^{\star}P < 0.05$, n = 9.

Aβ(1–42) (Pastorino et al., 2006). APP is a substrate for Pin1, which catalyzes a structural conformation of phospho-Ser/Thr-Pro for subsequent dephosphorylation by phosphatases. In AD brain and models, the Thr668-Pro motif has been shown to be increasingly phosphorylated, leading to increased Aβ production (Lee et al., 2003; Pastorino et al., 2006) and possibly suggesting Pin1 impairment. By using proteomics, studies from our laboratory have observed decreased levels and/or oxidation of this protein in MCI (Butterfield et al., 2006b) and AD (Sultana et al., 2006a, 2007a) brain. Consistent with the notion of Pin1 involvement in APP processing, we observed in the current study decreased levels of Pin1 in APP/PS-1 mice brain from both age groups (Fig. 1). Decreased levels of this protein in APP/PS-1 mice brain may contribute to the increased Aβ load. NAC administration to APP/PS-1 mice in both age groups caused a slight elevation of Pin1 levels compared with APP/PS-1 mice given water devoid of NAC; however, the result was not significant in brains from either the 9- or the 12-month-old mice. Although not entirely clear, it is possible that the diminished levels of Pin1 in APP/PS-1 mice are the result of oxidative damage by ROS produced by Aβ(1–42) and subsequent degradation of oxidized Pin1 by the 20S proteasome; this may also explain why a slight (although not significant) increase in Pin1 levels was observed in APP/PS-1 mice given NAC compared with APP/PS-1 mice given drinking water. More studies are needed to prove or refute this point decisively.

The oxidation of proteins by ROS may be responsible for damaging enzymes critical to neuronal function (Varadarajan et al., 2000; Castegna et al., 2003). By using proteomics, researchers in our laboratory identified brain proteins that are significantly oxidized in AD and MCI (Castegna et al., 2002a,b, 2003; Sultana et al., 2006a,b, 2007a,b; Butterfield et al., 2006a,b, 2007a; Perluigi, 2009; Reed, 2009). The oxidative modification of proteins either by introduction of a carbonyl functional-

ity or by nitration of tyrosine residues leads to alterations in protein tertiary structure, which typically results in altered activity (Butterfield and Stadtman, 1997; Stadtman and Berlett, 1997). In the present study, we showed the ability of in vivo NAC to reduce protein oxidation as indexed by decreased protein carbonyls (Fig. 2A,B), decreased protein-bound HNE (Fig. 3A,B), and decreased 3NT (Fig. 4A,B) in brain isolated from APP/PS-1 mice at 9 months of age, and, to a lesser degree, from 12-month-old mice. The current data are consistent with the notion that elevation of GSH via NAC (Pocernich et al., 2000, 2001) significantly modulates Aβ(1–42)-mediated protein oxidation in this model of MCI or early AD. This effect is more pronounced if given prior to significant Aβ deposition.

Elevated levels of lipid peroxidation products, such as HNE and acrolein, are observed in AD brain and in MCI (Sayre et al., 1997; Markesbery and Lovell, 1998; Lauderback et al., 2001; Mohmmad Abdul et al., 2006; Reed, 2009; Perluigi, 2009). HNE and other reactive alkenals can bind to proteins and render them inactive (Lauderback et al., 2001). In the present study, for both 9- and 12-month-old mice, we observed significantly increased protein-bound HNE in brain of APP/PS-1 mice relative to that of WT mice (Fig. 3B). There was a significant reduction in protein-bound HNE levels in brain isolated from APP/PS-1 mice that were given NAC compared with APP/PS-1 mice given drinking water in the 9-month-old group (Fig. 3B). The increase in endogenous GSH levels following in vivo administration of NAC (Pocernich et al., 2000) may explain the decreased protein-bound HNE levels, insofar as NAC reacts avidly by Michael addition with alkenals such as HNE and acrolein (Pocernich et al., 2001). No significant difference was observed in levels of protein-bound HNE in the brains of 12-month-old APP/PS-1 mice given NAC relative to APP/PS-1 mice given drinking water without NAC (Fig. 3B). The inability of NAC to suppress significantly the increased levels of protein-bound HNE in the brains of 12-month-old APP/PS-1 mice may be due to large amounts of HNE being formed in the lipid bilayer as Aβ levels increase (Anantharaman et al., 2006), whereas GSH, in contrast, resides primarily in the cytosol. Also, because APP/PS-1 mice produce significantly higher levels of Aβ(1–42) at about 6 months of age, initiation of NAC treatment at 7 months might have been too late to prevent the observed increase in protein-bound HNE. This notion is supported by the results from the 9-month-old age group, which show that increased protein-bound HNE in APP/PS-1 mice brain is ameliorated with administration of NAC begun at 4 months of age for 5 months, or before the production of senile plaques. Reductions in the level of lipid peroxidation products indicate less oxidative stress.

Protein-bound 3NT has been observed to be increased in MCI, early AD, and AD brain (Smith et al., 1997; Castegna et al., 2003; Sultana et al., 2006b, 2007b; Butterfield et al., 2007b; Reed et al., 2009). 3NT is formed from the reaction of NO with superoxide, producing peroxynitrite, which reacts with CO_2 to form the nitrite radical that attacks the ortho position of tyrosine residues on proteins (Butterfield et al., 2007b). Nitration of tyrosine residues results in changes in conformation, possibly altering protein function. By using proteomics, researchers in our laboratory discovered several proteins that were increasingly nitrated in AD and early AD brain compared with controls (Sultana et al., 2006b; Reed et al., 2009). In the present study, we observed significantly increased levels of 3NT in brains of mice isolated from APP/PS-1 mice compared with brains isolated from WT mice in both the 9- and the 12-month-old age groups (Fig. 4A,B). Increased Aβ-generated ROS in brains of APP/PS-1 mice is likely the cause of the observed increased protein nitration. Oral administration of NAC significantly suppressed 3NT in APP/PS-1 mice brains compared with brains isolated from APP/PS-1 mice given normal drinking water. As hypothesized for protein-bound HNE fluctuations, the increase in endogenous brain GSH levels after administration of NAC conceivably could explain the decreased 3NT levels; however, the free thiol group of NAC also is capable of scavenging NO via N_2O_3 directly to produce a nitrosothiol. Therefore, the antioxidant protection afforded by NAC from protein nitration may be the result of multiple mechanisms in vivo.

Diminished levels or activities of antioxidant defense enzymes render biomolecules vulnerable to oxidative modifications, contributing to various CNS disorders, including AD. Several potential antioxidant compounds to prevent Aβ-induced toxicity are under investigation (Anderson and Luo, 1998; Behl, 2002; Grundman and Delaney, 2002; Boyd-Kimball et al., 2005). NAC is a superior intracellular antioxidant and precursor of GSH relative to cysteine, because of the N-acetylation modification that increases lipophilicity and mobility across cell membranes. Moreover, cysteine, given as a drug, is toxic. Previously, our laboratory showed that rodents injected i.p. with NAC had significantly increased levels of brain GSH and were protected from protein and lipid oxidation (Koppal et al., 1999; Pocernich et al., 2000). In the present study, we measured the levels and activities of key enzymes involved in GSH metabolism, GPx and GR, in brains isolated from APP/PS-1 and WT mice.

GPx protects neurons from oxidative stress by catalyzing the reduction of H_2O_2 at the expense of GSH (Sies, 1999; Hansen et al., 2005). In this study, we observed a significant decreased GPx level and activity, as well as decreased GR activity, in brain isolated from APP/PS-1 mice compared with WT mice in both age groups (Fig. 5). The decreased activity in brains of APP/PS-1 mice conceivably could be due to oxidation of these proteins by ROS generated by Aβ. Consistent with this notion, oral administration of NAC to APP/PS-1 mice significantly suppressed protein oxidation (Figs. 2, 3) and protein nitration (Fig. 4) and augmented GPx activity in brain compared with APP/PS-1 mice

that were given plain drinking water (Fig. 5). NAC-induced increased GSH would likely scavenge ROS generated by Aβ in these mice, preventing oxidation of proteins and, thereby, preserving their activities. GR activity in APP/PS-1 mice was rescued by NAC treatment only in the 9-month-old group, which may indicate that administration of antioxidants after development of plaques in these animals is unable to defend this protein from the high levels of ROS and protein oxidation. Abdul et al. (2008) recently showed that nitration and oxidation in APP/PS-1 mice increase as a function of age, which is consistent with this hypothesis. The fluctuation in levels of GPx observed in this study is not entirely clear; however, lower levels and activity of GPx in brain isolated from APP/PS-1 mice (compared with WT) confer reduced clearance of cytosolic H_2O_2 and, therefore, increased intracellular ROS. NAC increased the activity and expression of this enzyme in vivo, resulting in more clearance of H_2O_2 and less ROS-mediated protein oxidation and lipid peroxidation, as observed in this study. Decreased GR activity would lower the rate at which GSSG is recycled to the reduced form, thereby altering the GSH/GSSG ratio, which is critical to the antioxidant and redox status of a cell. No alterations in GR levels were observed in either age group, regardless of treatment. However, GR activity of WT mice was reduced by NAC treatment. This is entirely reasonable, insofar as elevated GSH via NAC would preclude the need to reduce GSSG back to GSH, so lower activity of GR in brains of WT mice treated with NAC was observed.

G6PDH is an intracellular enzyme involved in the pentose phosphate pathway, which catalyzes the conversion of glucose-6-phosphate to 6-phosphogluconolactone, producing NADPH as a byproduct. NADPH provides the reducing electrons in the conversion of GSSG to GSH by GR. In this study, we observed significantly increased activity of G6PDH in brains of APP/PS-1 mice relative to brains isolated from WT. An increased activity of this enzyme in APP/PS-1 mice brain relative to WT suggests increased NADPH production, which favors the reduction of GSSG to GSH (Bolanos et al., 2008), possibly indicating cross-talk between these pathways. That GR activity was not fully restored to the level of WT mice at 9 or 12 months of age in NAC-treated APP/PS-1 mice suggests that elevated NADPH and elevated GSH were not sufficient to overcome Aβ-mediated loss of activity. Increased NADPH may also serve as a defense mechanism for preserving the reducing environment of a cell. However, increased activity of this enzyme in brains of APP/PS-1 mice also could be the result of activation by NO stress, which is feasible considering that we observed significant increases in 3NT in both age groups of APP/PS-1 mice (Fig. 4) as well as a slight decrease in activity of G6PDH in APP/PS-1 mice treated with NAC (though not significant; Fig. 7; Bolanos et al., 2004). Increased NADPH can also stimulate NADPH oxidases, which would increase intracellular superoxide ($O_2^{\cdot-}$) content. Therefore, the increased activity of this enzyme could have several downstream effects related to oxidative stress.

The ability of in vivo NAC to prevent endogenous oxidative stress, likely resulting from increased Aβ(1–42), in the APP/PS-1 mouse model of AD could also be related to its other properties, such as its antiinflammatory activity and/or its ability to activate several other protective signaling pathways (Zafarullah et al., 2003). Thus, we suggest that multiple biological functions of NAC could potentially contribute to counteract Aβ-driven neurotoxicity in the APP/PS-1 mice brain. However, its antioxidant capability and role as a GSH precursor clearly are important. Consistent with this notion, we showed decreased protein oxidation, nitration, and lipid peroxidation in APP/PS-1 mice orally administered NAC in vivo. Extrapolation of the results of the current study to subjects with MCI and AD suggests that earlier administration of NAC has protective advantages for treatment of oxidative stress-mediated neurodegenerative disorders compared with treatment at a later age. This observation has implications for the therapy of AD. In general, AD clinical trials using antioxidants have been disappointing (Boothby and Doering, 2005). Even in MCI, vitamin E reportedly did not slow conversion to AD (Petersen et al., 2005). Nevertheless, the current study suggests that, begun early enough, defense against oxidative stress in vivo by elevation of an endogenous antioxidant might have promise in slowing progression of AD. Studies to test this notion and to elucidate further the mechanistic aspects of neuroprotection implied by lower oxidative stress following oral administration of NAC in the APP/PS-1 mouse model of MCI and AD are in progress.

ACKNOWLEDGMENT

We thank Cephalon for the generous gift of the mice.

REFERENCES

Abdul HM, Sultana R, St. Clair DK, Markesbery WR, Butterfield DA. 2008. Oxidative damage in brain from human mutant APP/PS-1 double knock-in mice as a function of age. Free Radic Biol Med 45:1420–1425.

Anantharaman M, Tangpong J, Keller JN, Murphy MP, Markesbery WR, Kiningham KK, St. Clair DK. 2006. Beta-amyloid mediated nitration of manganese superoxide dismutase: implication for oxidative stress in a APPNLH/NLH X PS-1P264L/P264L double knock-in mouse model of Alzheimer's disease. Am J Pathol 168:1608–1618.

Anderson ME, Luo JL. 1998. Glutathione therapy: from prodrugs to genes. Semin Liver Dis 18:415–424.

Andreassen OA, Dedeoglu A, Klivenyi P, Beal MF, Bush AI. 2000. N-acetyl-L-cysteine improves survival and preserves motor performance in an animal model of familial amyotrophic lateral sclerosis. Neuroreport 11:2491–2493.

Ansari MA, Joshi G, Huang Q, Opii WO, Abdul HM, Sultana R, Butterfield DA. 2006. In vivo administration of D609 leads to protection of subsequently isolated gerbil brain mitochondria subjected to in vitro oxidative stress induced by amyloid beta-peptide and other oxidative stressors: relevance to Alzheimer's disease and other oxidative stress-

related neurodegenerative disorders. Free Radic Biol Med 41:1694–1703.

Beckman KB, Ames BN. 1998. The free radical theory of aging matures. Physiol Rev 78:547–581.

Behl C. 2002. Neuroprotective strategies in Alzheimer's disease. Adv Exp Med Biol 513:475–496.

Benzi G, Moretti A. 1995. Are reactive oxygen species involved in Alzheimer's disease? Neurobiol Aging 16:661–674.

Bolanos JP, Cidad P, Garcia-Nogales P, Delgado-Esteban M, Fernandez E, Almeida A. 2004. Regulation of glucose metabolism by nitrosative stress in neural cells. Mol Aspects Med 25:61–73.

Bolanos JP, Delgado-Esteban M, Herrero-Mendez A, Fernandez-Fernandez S, Almeida A. 2008. Regulation of glycolysis and pentose-phosphate pathway by nitric oxide: impact on neuronal survival. Biochim Biophys Acta 1777:789–793.

Boothby LA, Doering PL. 2005. Vitamin C and vitamin E for Alzheimer's disease. Ann Pharmacother 39:2073–2080.

Borchelt DR, Thinakaran G, Eckman CB, et al. 1996. Familial Alzheimer's disease-linked presenilin 1 variants elevate Abeta1–42/1–40 ratio in vitro and in vivo. Neuron 17:1005–1013.

Borchelt DR, Ratovitski T, van Lare J, Lee MK, Gonzales V, Jenkins NA, Copeland NG, Price DL, Sisodia SS. 1997. Accelerated amyloid deposition in the brains of transgenic mice coexpressing mutant presenilin 1 and amyloid precursor proteins. Neuron 19:939–945.

Bosetti F, Brizzi F, Barogi S, Mancuso M, Siciliano G, Tendi EA, Murri L, Rapoport SI, Solaini G. 2002. Cytochrome c oxidase and mitochondrial F1F0-ATPase (ATP synthase) activities in platelets and brain from patients with Alzheimer's disease. Neurobiol Aging 23:371–376.

Boyd-Kimball D, Sultana R, Poon HF, Mohmmad-Abdul H, Lynn BC, Klein JB, Butterfield DA. 2005. Gamma-glutamylcysteine ethyl ester protection of proteins from Abeta(1–42)-mediated oxidative stress in neuronal cell culture: a proteomics approach. J Neurosci Res 79:707–713.

Butterfield DAP. 2002. Elevated gutathione as a therapeutic strategy in Alzheimer's disease. Drug Dev Res 56:428–437.

Butterfield DA, Lauderback CM. 2002. Lipid peroxidation and protein oxidation in Alzheimer's disease brain: potential causes and consequences involving amyloid beta-peptide-associated free radical oxidative stress. Free Radic Biol Med 32:1050–1060.

Butterfield D, Stadtman ER. 1997. Protein oxidation processes in aging brain. Adv Cell Aging Gerontol 2:161–191.

Butterfield DA, Drake J, Pocernich C, Castegna A. 2001. Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid beta-peptide. Trends Mol Med 7:548–554.

Butterfield D, Castegna A, Pocernich C, Drake J, Scapagnini G, Calabrese V. 2002. Nutritional approaches to combat oxidative stress in Alzheimer's disease. J Nutr Biochem 13:444.

Butterfield DA, Gnjec A, Poon HF, Castegna A, Pierce WM, Klein JB, Martins RN. 2006a. Redox proteomics identification of oxidatively modified brain proteins in inherited Alzheimer's disease: an initial assessment. J Alzheimers Dis 10:391–397.

Butterfield DA, Poon HF, St. Clair D, Keller JN, Pierce WM, Klein JB, Markesbery WR. 2006b. Redox proteomics identification of oxidatively modified hippocampal proteins in mild cognitive impairment: insights into the development of Alzheimer's disease. Neurobiol Dis 22: 223–232.

Butterfield DA, Reed T, Perluigi M, De Marco C, Coccia R, Cini C, Sultana R. 2006c. Elevated protein-bound levels of the lipid peroxidation product, 4-hydroxy-2-nonenal, in brain from persons with mild cognitive impairment. Neurosci Lett 397:170–173.

Butterfield DA, Reed T, Newman SF, Sultana R. 2007a. Roles of amyloid beta-peptide-associated oxidative stress and brain protein modifications in the pathogenesis of Alzheimer's disease and mild cognitive impairment. Free Radic Biol Med 43:658–677.

Butterfield DA, Reed TT, Perluigi M, De Marco C, Coccia R, Keller JN, Markesbery WR, Sultana R. 2007b. Elevated levels of 3-nitrotyrosine in brain from subjects with amnestic mild cognitive impairment: implications for the role of nitration in the progression of Alzheimer's disease. Brain Res 1148:243–248.

Carlberg I, Mannervik B. 1985. Glutathione reductase. Methods Enzymol 113:484–490.

Castegna A, Aksenov M, Aksenova M, Thongboonkerd V, Klein JB, Pierce WM, Booze R, Markesbery WR, Butterfield DA. 2002a. Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part I: creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1. Free Radic Biol Med 33:562–571.

Castegna A, Aksenov M, Thongboonkerd V, Klein JB, Pierce WM, Booze R, Markesbery WR, Butterfield DA. 2002b. Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part II: dihydropyrimidinase-related protein 2, alpha-enolase and heat shock cognate 71. J Neurochem 82:1524–1532.

Castegna A, Thongboonkerd V, Klein JB, Lynn B, Markesbery WR, Butterfield DA. 2003. Proteomic identification of nitrated proteins in Alzheimer's disease brain. J Neurochem 85:1394–1401.

Dringen R, Hamprecht B. 1999. N-acetylcysteine, but not methionine or 2-oxothiazolidine-4-carboxylate, serves as cysteine donor for the synthesis of glutathione in cultured neurons derived from embryonal rat brain. Neurosci Lett 259:79–82.

Farr SA, Poon HF, Dogrukol-Ak D, Drake J, Banks WA, Eyerman E, Butterfield DA, Morley JE. 2003. The antioxidants alpha-lipoic acid and N-acetylcysteine reverse memory impairment and brain oxidative stress in aged SAMP8 mice. J Neurochem 84:1173–1183.

Fontaine MA, Geddes JW, Banks A, Butterfield DA. 2000. Effect of exogenous and endogenous antioxidants on 3-nitropionic acid-induced in vivo oxidative stress and striatal lesions: insights into Huntington's disease. J Neurochem 75:1709–1715.

Grundman M, Delaney P. 2002. Antioxidant strategies for Alzheimer's disease. Proc Nutr Soc 61:191–202.

Halliwell B. 2001. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs Aging 18: 685–716.

Hansen JM, Go YM, Jones DP. 2005. Nuclear and mitochondrial compartmentation of oxidative stress and redox signaling. Annu Rev Pharmacol Toxicol 45:1–25.

Hensley K, Hall N, Subramaniam R, et al. 1995. Brain regional correspondence between Alzheimer's disease histopathology and biomarkers of protein oxidation. J Neurochem 65:2146–2156.

Katzman R, Saitoh T. 1991. Advances in Alzheimer's disease. FASEB J 5:278–286.

Keller JN, Schmitt FA, Scheff SW, Ding Q, Chen Q, Butterfield DA, Markesbery WR. 2005. Evidence of increased oxidative damage in subjects with mild cognitive impairment. Neurology 64:1152–1156.

Koppal T, Drake J, Butterfield DA. 1999. In vivo modulation of rodent glutathione and its role in peroxynitrite-induced neocortical synaptosomal membrane protein damage. Biochim Biophys Acta 1453:407–411.

Lauderback CM, Hackett JM, Huang FF, Keller JN, Szweda LI, Markesbery WR, Butterfield DA. 2001. The glial glutamate transporter, GLT-1, is oxidatively modified by 4-hydroxy-2-nonenal in the Alzheimer's disease brain: the role of Abeta1–42. J Neurochem 78:413–416.

Lee MS, Kao SC, Lemere CA, Xia W, Tseng HC, Zhou Y, Neve R, Ahlijanian MK, Tsai LH. 2003. APP processing is regulated by cytoplasmic phosphorylation. J Cell Biol 163:83–95.

Lovell MA, Ehmann WD, Butler SM, Markesbery WR. 1995. Elevated thiobarbituric acid-reactive substances and antioxidant enzyme activity in the brain in Alzheimer's disease. Neurology 45:1594–1601.

Lovell MA, Gabbita SP, Markesbery WR. 1999. Increased DNA oxidation and decreased levels of repair products in Alzheimer's disease ventricular CSF. J Neurochem 72:771–776.

Marcus DL, Thomas C, Rodriguez C, Simberkoff K, Tsai JS, Strafaci JA, Freedman ML. 1998. Increased peroxidation and reduced antioxidant enzyme activity in Alzheimer's disease. Exp Neurol 150:40–44.

Markesbery WR. 1997. Oxidative stress hypothesis in Alzheimer's disease. Free Radic Biol Med 23:134–147.

Markesbery WR, Lovell MA. 1998. Four-hydroxynonenal, a product of lipid peroxidation, is increased in the brain in Alzheimer's disease. Neurobiol Aging 19:33–36.

Mecocci P, MacGarvey U, Kaufman AE, Koontz D, Shoffner JM, Wallace DC, Beal MF. 1993. Oxidative damage to mitochondrial DNA shows marked age-dependent increases in human brain. Ann Neurol 34:609–616.

Mohmmad Abdul H, Sultana R, Keller JN, St. Clair DK, Markesbery WR, Butterfield DA. 2006. Mutations in amyloid precursor protein and presenilin-1 genes increase the basal oxidative stress in murine neuronal cells and lead to increased sensitivity to oxidative stress mediated by amyloid beta-peptide (1–42), HO and kainic acid: implications for Alzheimer's disease. J Neurochem 96:1322–1335.

Nunomura A, Perry G, Pappolla MA, Wade R, Hirai K, Chiba S, Smith MA. 1999. RNA oxidation is a prominent feature of vulnerable neurons in Alzheimer's disease. J Neurosci 19:1959–1964.

Pahan K, Sheikh FG, Namboodiri AM, Singh I. 1998. N-acetyl cysteine inhibits induction of no production by endotoxin or cytokine stimulated rat peritoneal macrophages, C6 glial cells and astrocytes. Free Radic Biol Med 24:39–48.

Pastorino L, Sun A, Lu PJ, et al. 2006. The prolyl isomerase Pin1 regulates amyloid precursor protein processing and amyloid-beta production. Nature 440:528–534.

Perluigi MSR, Cenini G, Di Domenico F, Memo M, Pierce WM, Coccia R, Butterfield DA. 2009. Redox proteomics identification of HNE-modified brain proteins in Alzheimer's disease: role of lipid peroxidation in Alzheimer's disease pathogenesis. Proteom Clin Appl 3: 682–693.

Petersen RC, Thomas RG, Grundman M, et al. 2005. Vitamin E and donepezil for the treatment of mild cognitive impairment. N Engl J Med 352:2379–2388.

Pocernich CB, La Fontaine M, Butterfield DA. 2000. In-vivo glutathione elevation protects against hydroxyl free radical-induced protein oxidation in rat brain. Neurochem Int 36:185–191.

Pocernich CB, Cardin AL, Racine CL, Lauderback CM, Butterfield DA. 2001. Glutathione elevation and its protective role in acrolein-induced protein damage in synaptosomal membranes: relevance to brain lipid peroxidation in neurodegenerative disease. Neurochem Int 39:141–149.

Prescott LF, Park J, Ballantyne A, Adriaenssens P, Proudfoot AT. 1977. Treatment of paracetamol (acetaminophen) poisoning with N-acetylcysteine. Lancet 2:432–434.

Reaume AG, Howland DS, Trusko SP, Savage MJ, Lang DM, Greenberg BD, Siman R, Scott RW. 1996. Enhanced amyloidogenic processing of the beta-amyloid precursor protein in gene-targeted mice bearing the Swedish familial Alzheimer's disease mutations and a ''humanized'' Abeta sequence. J Biol Chem 271:23380–23388.

Reed TT, Pierce WM, Turner DM, Markesbery WR, Butterfield DA. 2009. Proteomic identification of nitrated brain proteins in early Alzheimer's disease inferior parietal lobule. J Cell Mol Med 13:2019–2029.

Sano M, Ernesto C, Thomas RG, et al. 1997. A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. N Engl J Med 336:1216–1222.

Sayre LM, Zelasko DA, Harris PL, Perry G, Salomon RG, Smith MA. 1997. 4-Hydroxynonenal-derived advanced lipid peroxidation end products are increased in Alzheimer's disease. J Neurochem 68:2092–2097.

Sies H. 1999. Glutathione and its role in cellular functions. Free Radic Biol Med 27:916–921.

Siman R, Reaume AG, Savage MJ, Trusko S, Lin YG, Scott RW, Flood DG. 2000. Presenilin-1 P264L knock-in mutation: differential effects on abeta production, amyloid deposition, and neuronal vulnerability. J Neurosci 20:8717–8726.

Smith MA, Richey Harris PL, Sayre LM, Beckman JS, Perry G. 1997. Widespread peroxynitrite-mediated damage in Alzheimer's disease. J Neurosci 17:2653–2657.

Stadtman ER, Berlett BS. 1997. Reactive oxygen-mediated protein oxidation in aging and disease. Chem Res Toxicol 10:485–494.

Sultana R, Boyd-Kimball D, Poon HF, et al. 2006a. Oxidative modification and down-regulation of Pin1 in Alzheimer's disease hippocampus: a redox proteomics analysis. Neurobiol Aging 27:918–925.

Sultana R, Poon HF, Cai J, Pierce WM, Merchant M, Klein JB, Markesbery WR, Butterfield DA. 2006b. Identification of nitrated proteins in Alzheimer's disease brain using a redox proteomics approach. Neurobiol Dis 22:76–87.

Sultana R, Boyd-Kimball D, Cai J, Pierce WM, Klein JB, Merchant M, Butterfield DA. 2007a. Proteomics analysis of the Alzheimer's disease hippocampal proteome. J Alzheimers Dis 11:153–164.

Sultana R, Reed T, Perluigi M, Coccia R, Pierce WM, Butterfield DA. 2007b. Proteomic identification of nitrated brain proteins in amnestic mild cognitive impairment: a regional study. J Cell Mol Med 11:839–851.

Sultana R, Piroddi M, Galli F, Butterfield DA. 2008. Protein levels and activity of some antioxidant enzymes in hippocampus of subjects with amnestic mild cognitive impairment. Neurochem Res 33:2540–2546.

van Groen T, Kiliaan AJ, Kadish I. 2006. Deposition of mouse amyloid beta in human APP/PS1 double and single AD model transgenic mice. Neurobiol Dis 23:653–662.

Varadarajan S, Yatin S, Aksenova M, Butterfield DA. 2000. Review: Alzheimer's amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity. J Struct Biol 130:184–208.

Wang J, Xiong S, Xie C, Markesbery WR, Lovell MA. 2005. Increased oxidative damage in nuclear and mitochondrial DNA in Alzheimer's disease. J Neurochem 93:953–962.

Wheeler CR, Salzman JA, Elsayed NM, Omaye ST, Korte DW Jr. 1990. Automated assays for superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activity. Anal Biochem 184:193–199.

Yan CY, Greene LA. 1998. Prevention of PC12 cell death by N-acetylcysteine requires activation of the Ras pathway. J Neurosci 18:4042–4049.

Zafarullah M, Li WQ, Sylvester J, Ahmad M. 2003. Molecular mechanisms of N-acetylcysteine actions. Cell Mol Life Sci 60:6–20.

Zhang Q, Tian H, Fu X, Zhang G. 2003. Delayed activation and regulation of MKK7 in hippocampal CA1 region following global cerebral ischemia in rats. Life Sci 74:37–45.